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Characterization of West European field isolates and vaccine strains of avian infectious laryngotracheitis virus by restriction fragment length polymorphism and sequence analysis

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Characterization of West European field isolates and vaccine strains of avian infectious laryngotracheitis virus by restriction fragment length polymorphism and sequence analysis

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SUMMARY

Infectious laryngotracheitis (ILT) is a dramatic disease of the upper respiratory tract in poultry caused by a herpesvirus. In this study, we investigated the characteristics of West European field isolates of *infectious laryngotracheitis virus (ILTV)* to gain more information on their diversity.

The examined 104 isolates, collected from acute outbreaks during the last 35 years, originated from eight different countries: Switzerland (48), Germany (21), Sweden (14), the United Kingdom (9), Italy (5), Belgium (4), Austria (2) and Norway (1). Two vaccines, a chicken embryo origin (CEO) product and a tissue culture origin (TCO) product, were included in the survey. Polymerase chain reaction (PCR) was performed to amplify a 2.1 kb DNA fragment of *ILTV* using primers generated for the *thymidine kinase (TK)* gene. After digestion of the resulting PCR products by restriction endonuclease (RE) *Hae*III, restriction fragment length polymorphism (RFLP) analysis was carried out. PCR amplicons of three field isolates and both vaccine strains were selected for sequencing.

98 field isolates showed the same cleavage pattern and were identical to both vaccine strains (clone 1). They differed from five Swiss isolates with identical cleavage pattern (clone 2) and one Swedish isolate (clone 3).

The present study demonstrated that at least three clones of *ILTV* have been circulating in Western Europe during the last 35 years. The 104 isolates analyzed showed a high genetic similarity regarding the *TK* gene and a large majority of the field isolates (98/104) was genetically related to the vaccine strains.

Key words: Europe, *infectious laryngotracheitis virus,* polymerase chain reaction, restriction fragment length polymorphism, sequence, Switzerland, *thymidine kinase,* vaccine

Abbreviations: CEO = chicken embryo origin, *GaHV-1 = gallid herpesvirus 1,* ILT = infectious laryngotracheitis, *ILTV = infectious laryngotracheitis virus,* ORF = open reading frame, PCR = polymerase chain reaction, RE = restriction endonuclease, RFLP = restriction fragment length polymorphism, TCO = tissue culture origin, *TK = thymidine kinase*

INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a respiratory disease of chickens. It can also affect pheasants and peacocks. Difficulty in breathing, coughing up of blood or bloody mucus, swollen orbital sinuses, high morbidity (up to 100%), variable mortality and decreased egg production in laying hens are characteristic of the acute form of ILT. Chronic ILT may look like any other respiratory infection (1). The causative agent is *Infectious laryngotracheitis virus (ILTV),* also designated *Gallid herpesvirus 1 (GaHV-1),* which belongs to the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (23). The genome of *ILTV* is a linear double-stranded DNA of approximately 155 kb in size (15,20). Like other herpesviruses, *ILTV* induces latent infections, mainly in the trigeminal ganglion (3,27). Latently infected chickens are the primary source of ILT outbreaks.

In Switzerland, ILT is a notifiable disease since 1987. Every year five to twenty outbreaks are registered. They mainly occur in small, backyard and fancy breed flocks and occasionally in commercial chickens (13,14). Although live attenuated vaccines against ILT are available in Europe, their application is not allowed in Switzerland. The reasons for the ban of vaccination are mainly economical such as the negligible number of outbreaks, the difficult diagnosis of infections in vaccinated birds and the tedious differentiation between field and vaccine strains. In the majority of European countries vaccination against ILT is common practice in regions with repeated ILT outbreaks, although it is known that live attenuated vaccines possess considerable residual virulence, which may increase after bird-to-bird passage (10).

Due to their ability to detect acute as well as latent infections, molecular methods have proven to be more sensitive than virus isolation in the identification process of *ILTV* (2,16,18,26). Detection of DNA by conventional or real-time polymerase chain reaction (PCR) technique followed by restriction fragment length polymorphism (RFLP) analysis is

the preferred method for differentiation between field isolates and vaccine strains of *ILTV* (4,5,6,7,8,11,19,21,22,25). These techniques combined with DNA sequence analysis or cluster analyses of RFLP patterns of *ILTV* were used for epidemiological investigations and phylogenetic studies as reported previously (6,11,19,21,22).

Han and Kim (12) compared several *ILTV* strains from Korean layer hens that experienced respiratory signs and mortality between 1982 and 1998 by examining RFLP patterns of the amplified *TK* gene and by virulence testing based on pathogenicity studies in chickens. Further DNA sequencing studies allowed the authors to differentiate between field isolates of high and low virulence (11). The presence of the amino acid threonine (base-triplet ACG) at position 252 in the sequence of the *TK* gene was an indication for strains of low virulence, whereas the amino acid methionine (base-triplet ATG) at the same position pointed to virulent strains. The same nucleotide and amino acid sequences as the low virulent strains exhibited at position 252 were previously published for the Thorne strain (GenBank accession number D00565) as well as for the strain 216 (GenBank accession number L36139) with 1092 bp in length and for the strain 632 (GenBank accession number S83714) with 1089 bp in length.

The objective of the present study was to characterize 104 *ILTV* isolates from vaccinated and unvaccinated fowl with acute ILT infection by PCR/RFLP in order to investigate the diversity of the strains in Western Europe during the last 35 years.

MATERIALS AND METHODS

Sources of *ILTV* isolates

The viruses compared in our study were isolated from natural acute outbreaks of ILT in Switzerland and seven further West European countries (Table 1). The analyzed 104 field isolates, collected over a period of 35 years (1973-2007), originated from vaccinated and unvaccinated fowl, covering different bird species and poultry production sectors. Forty-eight isolates were collected in Switzerland, including four isolates from commercial chicken flocks and 44 isolates from hobby or fancy chicken flocks, which are often mixed with pheasants, peacocks, quails or other exotic species. Fifty-six isolates were from diagnosed ILT cases in Germany (21), Sweden (14), the United Kingdom (9), Italy (5), Belgium (4), Austria (2) and Norway (1). Irrespective of differing national control programs, such as ban of vaccination (Switzerland, Sweden, Norway), eradication of affected flocks (Switzerland, Sweden, Norway), selected vaccination of commercial poultry (Belgium, Italy) or use of vaccines in both commercial and non-commercial poultry (Germany, United Kingdom, Austria) all isolates originated from clinical outbreaks. Complete information about epidemiology was only available for a part of the ILT cases; mainly it was difficult to collect reliable data on the vaccination status of the investigated birds (Table 1). Two ILT vaccines, Serva strain and Samberg strain, were of chicken embryo origin (CEO) (Nobilis®ILT, Intervet International B.V. Boxmeer, The Netherlands) and tissue culture origin (TCO) (LT-Ivax®, Schering-Plough Animal Health San Augustin, Spain). The viral DNA was isolated directly from the lyophilized vaccine powder. *ILTV* DNA of two field isolates (TW/90 and TW/91-1) and two vaccine strains (CEO and TCO) from Taiwan served as reference material (4).

Extraction of *ILTV* DNA

Preparation of *ILTV* DNA was based on the procedures described by a standard protocol of Sambrook et al. (24). 400 µl of each isolate were treated with 15 µl proteinase K (Roche), 22.5 µl sodium dodecyl sulfate (SDS) and 22.5 µl TEN buffer (Tris 100 mM, EDTA 10 mM, NaCl 1 M, pH 7.4) at 56°C for two hours. The DNA was extracted twice with an equal volume of phenol, purified twice with an equal volume of isoamylalcohol-chloroform (1:24), precipitated at -80°C for 30 min in 1/10 volume of 3 M sodium acetate (pH 4.8) and two volumes of cold (-20°C) absolute ethanol, and then pelleted by centrifugation at 13.000 rpm at 4°C for 30 min. The pellet was rinsed in cold (-20°C) 70% ethanol, centrifuged at 13.000 rpm at 4°C for 15 min, air dried for 60 min, resuspended in 20 µl demineralized water, and stored at 4°C overnight. Serial tenfold dilutions of final DNA preparation were prepared in demineralized water. From each original isolate the extraction of *ILTV* DNA was additionally carried out using the QuickGene DNA tissue kit S developed from FUJIFILM Corporation (Bucher Biotec, Switzerland) according to the manufacturer's instructions. 1 µl DNA template of each isolate obtained from the two different extraction methods was used for PCR. Hence, PCR/RFLP was performed twice for all 104 samples. The reproducibility of the techniques could be verified.

Primer design

PCR oligonucleotide sense and antisense primers were designed by Microsynth (Microsynth, Switzerland) on the basis of the published DNA sequence of the Thorne strain of *ILTV* (GenBank accession number D00565). The primers were used to amplify a 2.1 kb fragment of the *ILTV* genome containing the *TK* gene. The primers designed were *TK* gene (sense) 5'-GCTGGGCTAAATCATCCAAG-3' and *TK* gene (antisense) 5'-GGAAGCGGAACATTACGAAC-3'.

Polymerase chain reaction (PCR)

Hot start PCR amplification was performed using a DNA thermocycler GeneAmp® PCR System 2400 (Perkin Elmer). The reaction mixture (25 µl) consisted of 1 µl DNA template and 24 µl master mix containing 1x PCR buffer with 1.5 mM $MgCl_2$, 200 µM of each dNTP, 200 mM of each primer, and HotStarTaq DNA polymerase (HotStarTaq Master Mix Kit, Qiagen). Phosphate-buffered saline (PBS) served as process control. The *ILTV* strain T 322/04 (isolate of a one year old cock with ILT infection diagnosed in 2004 by serology, histology and observed pock lesions after propagation in the chorioallantoic membrane (CAM) of embryonated chicken eggs) was used as a positive control. A negative control (master mix with demineralized water instead of template DNA) was also included. The condition for PCR reaction was: 35 cycles of 95°C for 15 min (activation of DNA polymerase), 94°C for 1 min (denaturation), 56°C for 30 s (annealing), 72°C for 1 min (extension), followed by 1 cycle of 72°C for 5 min (final extension). The samples were cooled to 4°C and stored at 4°C. Following amplification, 10 µl of each PCR product was examined by electrophoresis (70 V for 1 hour) in a 1% agarose gel (Agarose Standard Eurobio) in 1x TAE buffer (Tris-acetate 40 mM, EDTA 2 mM, pH 8.0) containing ethidium bromide (3 µl/100 ml). The size of PCR products was determined by comparison with a molecular weight marker (1 kb DNA ladder, BioConcept), and DNA bands were visualized under UV-illumination.

Restriction endonuclease (RE) digestion and restriction fragment length polymorphism (RFLP) analysis

15 µl of each amplification product was digested by 1 µl of tetranucleotide-recognizing RE *Hae*III (Roche) using 10 µl RE buffer M supplied by the manufacturer in a final volume of 100 µl at 37°C for one hour. The digested DNA was precipitated, pelleted by centrifugation, rinsed, centrifuged again, and air dried, as described above, resuspended in

10 µl demineralized water and stored at 4°C for 40 min. DNA fragments were separated by electrophoresis (70 V for 2 hours) in a 2% agarose gel (Agarose Standard Eurobio) in 1x TAE buffer (Tris-acetate 40 mM, EDTA 2 mM, pH 8.0) containing ethidium bromide (3 µl/100 ml) to stain the DNA. The size of DNA RE fragments was compared with a molecular weight marker (peqGOLD 100 bp DNA-Leiter Plus, PEQLAB, Germany) ranging from 100 bp to 3000 bp bands. Bands were visualized using UV-transillumination (Alpha Innotech, CA, USA) and photographed.

Sequencing

Sequencing of the 2.1 kb DNA fragment of the three field isolates CH04, CH95 and S04 with different RFLP patterns and the two vaccine strains was performed. The amplified PCR products were purified using High Pure PCR Product Purification Kit (Roche) according to manufacturer's instructions. Purified DNA was sent to Microsynth Corporation (Microsynth, Switzerland) for sequencing. The nucleotide sequences were determined for both strands, checking them five times. The sequences were assembled using software Lasergene SeqMan7.2 (DNASTAR). For highlighting open reading frames (ORF) and comparing the identity of the sequencing products, the sequences were analyzed using available computer software (http://seqtool.sdsc.edu/CGI/BW.cgi and http://www.ncbi.nlm.nih.gov/blast/). Alignment of the nucleotide and predicted amino acid sequences for the *TK* protein of the Thorne strain, the three field isolates CH04, CH95 and S04 and the two vaccine strains CEO and TCO were performed using EXPASY molecular tools (http://www.expasy.ch).

GenBank accession numbers

The *TK* sequences presented in this study have been submitted to GenBank under accession numbers ranging from EU360946 to EU360950.

RESULTS

PCR amplification of a 2.1 kb region of the *ILTV* genome containing the *TK* gene

All PCR products of the *ILTV* field isolates and vaccine strains analyzed in our study resulted in one DNA band of approximately 2.1 kb (Fig. 1). The PCR products of three field isolates of *ILTV* (two from Switzerland, one from Southern Germany) in comparison with molecular weight standards are presented in Fig. 1.

All *ILTV* isolates analyzed by use of PCR were detected. The primer set, generated for *TK* gene of the Thorne strain of *ILTV*, was shown to be specific for *ILTV*. In our preliminary studies it did not amplify other viruses from avian sources and in particular no other herpesviruses such as Marek's disease virus or pigeon herpesvirus.

Comparison of RFLP patterns generated by *Hae*III-digestion of the 2.1 kb PCR product

Three different RFLP patterns were observed (Fig. 2). Forty-three Swiss field isolates and 55 field isolates from the other seven West European countries as well as the two vaccine strains produced identical RFLP patterns (classified as clone 1; Fig. 2, lanes 1, 4 and 5). Twenty clone 1 field isolates originated with certainty from unvaccinated birds, 61 isolates were from birds which were highly suspicious for not being vaccinated and 17 isolates from birds with high suspicion for being vaccinated. Clone 1 spread among birds in Swiss backyard flocks (39/44) and in Swiss commercial chicken flocks (4/4) as well as in hobby poultry farming and the poultry industry in Germany (21/21), Sweden (13/14), the United Kingdom (9/9), Italy (5/5), Belgium (4/4), Austria (2/2) and Norway (1/1) during the last 35 years. Of the 98 clone 1 isolates 66 originated from backyard flocks and 32 were obtained from commercial poultry. No significance for clone 1 was found between commercial poultry and backyard flocks ($p > 0.05$, chi-square test). Five Swiss field isolates (clone 2; Fig. 2, lane 2) and one Swedish field isolate (clone 3; Fig. 2, lane 3) differed from the other field isolates and

the vaccine strains (clone 1; Fig. 2, lanes 1, 4 and 5). Only clone 1 has been circulating for 35 years in West European countries and was widespread in both poultry sectors, in commercial and in non-commercial poultry. The five Swiss clone 2 isolates from birds with high suspicion for not being vaccinated and the one Swedish clone 3 isolate from with certainty unvaccinated birds were limited either in regional spread or number of cases and therefore did not develop an epizootic character. Both clones were found in non-commercial poultry. Different clone patterns and origin of the field isolates and vaccine strains are presented in Table 2. Results of the positions of restriction sites for *Hae*III (GG:CC) of the sequences of the three isolates CH04, CH95 and S04 and the two vaccine strains CEO and TCO for the *TK* protein are presented in Table 3.

DNA sequence analysis of the 2.1 kb PCR product of three field isolates and two vaccine strains of *ILTV*

All strains selected for sequencing were 2167 bp in length. Computer analysis revealed the presence of one complete 1089 bp ORF for each amplicon and online BLAST search showed 99% nucleotide identity between the ORF of each of the five amplicons and the DNA sequence from the region of the *TK* gene of *ILTV* as determined by Griffin and Boursnell (9) from the Thorne strain which consists of 1092 bp. Results of the positions of nucleotide and amino acid changes of the sequences of the three isolates CH04, CH95 and S04 and the two vaccine strains CEO and TCO for the *TK* protein are presented in Table 3. The nucleotide sequences of the two vaccine strains were identical. The Swiss field isolate CH04 differed from the vaccine strains in one nucleotide at position 540 (C to T). This led to no change in amino acid. The same nucleotide substitution was found in the Swedish field isolate S04. Additionally, in the field isolate S04 another nucleotide mutation was present at position 411 (A to G) and led to the sequence GGCC and a unique RFLP pattern after digestion with *Hae*III (Fig. 2, lane 3). Again no change in amino acid occurred. The Swiss field isolate

CH95 differed from the vaccine strains by four nucleotides at position 123 (T to C), at position 141 (G to A), at position 594 (A to C) and at position 755 (C to T). Due to the change of A to C at position 594 an additional band was visible after *Hae*III-digestion and RFLP analysis (Fig. 2, lane 2). The nucleotide change at position 755 (C to T) caused a change in amino acid. Methionine (ATG) replaced threonine (ACG) at position 252 in the field isolate CH95.

DISCUSSION

In this study, we investigated the characteristics of 104 field isolates of *ILTV* from eight West European countries by PCR/RFLP and sequence analysis. The isolates, collected over a period of 35 years (1973-2007) showed three different cleavage patterns (clone 1, 2 and 3).

Molecular methods to identify and differentiate *ILTV* strains were developed by Clavijo and Nagy (5). They were able to separate a virulent field strain from three vaccine strains by use of PCR/RFLP. Using the same technique, Chang et al. (4) investigated field isolates collected from outbreaks of acute ILT in chickens in Taiwan and discriminated field isolates from vaccine strains. They were able to differentiate three CEO vaccines with identical RE cleavage products from two of four field isolates. The other two field isolates remained indistinguishable from the CEO vaccines but differed from a TCO vaccine. Their strains served as reference material and we could confirm the two different RFLP patterns and the diversity of the CEO and TCO vaccine strains. In our study, the TCO vaccine from Spain and the CEO vaccine from the Netherlands showed the same RFLP pattern (Fig. 2, lanes 4 and 5) and their nucleotide sequences were identical. They matched the virulent field strain 632 (GenBank accession number S83714), but differed from the isolate CH04 and the isolate S04 in one and two nucleotides, respectively. Han and Kim (11) also analyzed six commercial vaccines originating from chicken embryo and reported that the RFLP patterns of the *TK* gene of low virulence strains were identical to those of the vaccine strains. According to their sequence analyses of the low virulence and the vaccine strains, threonine was the amino acid at position 252. In our study, sequencing of the *TK* gene of the Swiss field isolate CH95 resulted in four nucleotides differing from the two vaccine strains (CEO and TCO). Interestingly, the changes in nucleotides were identical to three strains sequenced and specified as virulent by Han and Kim (11), including Korean field isolates of *ILTV* from vaccinated and unvaccinated chickens.

Our study showed that the *TK* gene of field clone 1 and clone 3 viruses (CH04 and S04) shared the amino acid threonine at position 252 with Korean field isolates previously identified as low virulence strains. On the other hand, the amino acid change in field clone 2 viruses (CH95) with a methionine at position 252 was equivalent with virulent strains from Korea. The sequence of vaccine clone 1 viruses with a threonine at position 252 matched sequences of *ILTV* strains either of high virulence, such as strain 632 from the United States as published by Keeler et al. (17) and of low virulence, such as Korean strains as described by Han and Kim (11). However, the sequence analysis of the *TK* gene of *ILTV* alone cannot be used to differentiate between *ILTV* of high and low virulence. It is highly likely that other in this study unexamined genes in the large genome of *ILTV* play a role in virulence.

Although we investigated a considerable number of Swiss *ILTV* isolates (48 isolates) covering different bird species and poultry production sectors the full comparison of the RE cleavage products showed only two different RFLP patterns. Forty-three out of 48 isolates from Switzerland belonged to clone 1. Five Swiss isolates could be grouped to clone 2, which was found repeatedly in fancy breed flocks between 1973 and 1995, but never detected in commercial poultry. Some clone 1 isolates from Switzerland may have also originated from illegally imported vaccinated backyard chickens of surrounding neighboring countries such as Germany and France. Therefore, we decided to analyze approximately an equal number of *ILTV* isolates from other European countries (56 isolates). Clone 1 was found in 55 isolates, clone 2 was not detected and clone 3 was only isolated from a Swedish non-commercial poultry of an outbreak in 2004. In the isolates analyzed (n = 104), originating from backyard flocks (n = 72) and commercial poultry (n = 32), clone 1 was equally represented with 92% (66/72) and 100% (32/32), respectively. In our study, the two vaccine strains from different origins produced RFLP patterns of clone 1.

Vaccination status of the birds from which *ILTV* was isolated and analyzed is shown in Table 1. As has been proved, only 21 of all examined 104 isolates and only 20 out of the 98

(20%) field clone 1 isolates with certainty came from birds of unvaccinated flocks. We speculate that some of the field clone 1 viruses from flocks of uncertain vaccination status were re-isolated vaccines, especially based on the fact that vaccination is allowed in other West European countries surrounding Switzerland.

It is a fact that new clones, such as clone 2 and clone 3 in our study, primarily originated from non-commercial poultry. The latency and longer circulation of the virus in non-commercial poultry seems to favor the development of new clones, whereas in commercial poultry quicker and/or stricter control measures such as eradication, vaccination, all in-, all out-management limit the duration of viral circulation in birds.

The present study showed that at least three clones of *ILTV* have been circulating in Western Europe during the last 35 years. The most relevant part of this study was that a large majority of the West European field isolates analyzed, 98 out of 104 isolates, were genetically related to the vaccine strains.

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Fig. 1.

Agarose gel electrophoresis of PCR amplification products of two ILT isolates from Switzerland and one ILT isolate from Southern Germany, with primers specific to the *TK* gene of *ILTV*. PCR was performed as described in the text. Lane M: 1 kb DNA ladder (bands of 500, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000 and 10000 bp are shown in the far left lane), lanes 1 and 2: ILT field isolates from Switzerland, lane 3: ILT field isolate from Southern Germany, lane 4: process control, lane 5: positive control and lane 6: negative control.

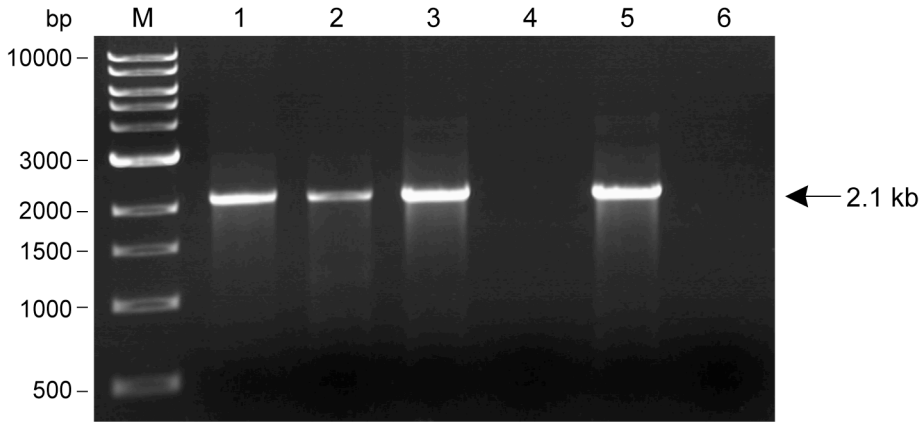

Fig. 1

Fig. 2.

Agarose gel electrophoresis of DNA RE fragments of the 2.1 kb PCR amplification product of three ILT field isolates and two ILT vaccine strains, digested by RE *Hae*III. RFLP analysis was performed as described in the text. Lane M: 100 bp DNA ladder (bands of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 3000 bp are shown in the far left lane), lane 1: ILT field isolate CH04, lane 2: ILT field isolate CH95, lane 3: ILT field isolate S04, lane 4: ILT vaccine strain CEO and lane 5: ILT vaccine strain TCO, digested by *Hae*III.

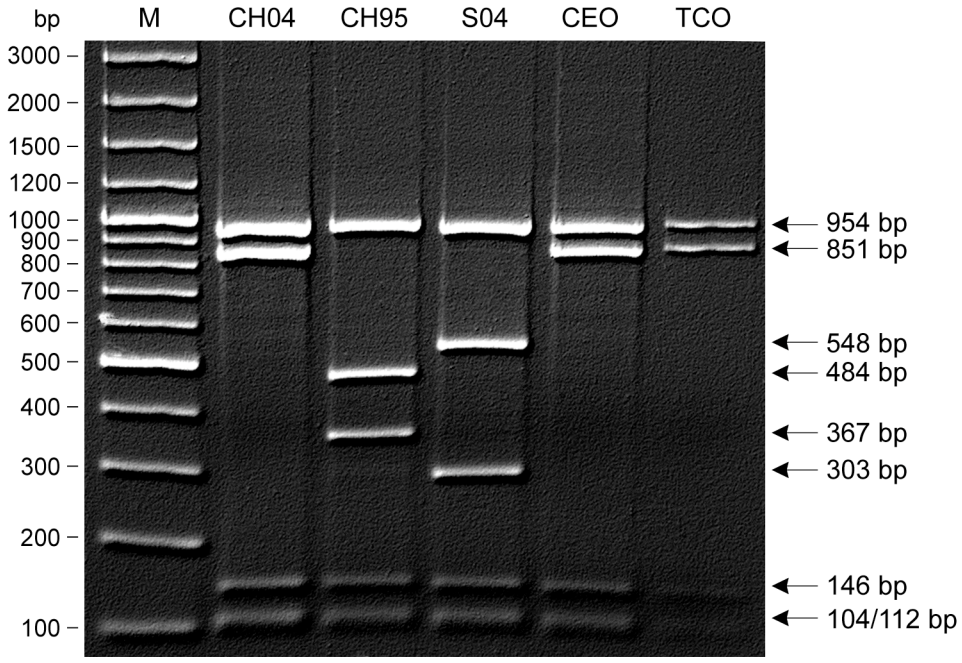

Fig. 2

Table 1. Origin, number, year of isolation, husbandry and vaccination status of *ILTV* isolates analyzed.

Country	Number of isolates	Year of isolation	Husbandry	
			Non-commercial poultry	Commercial poultry
Switzerland	48	1973 - 2007	44[B]	4[A]
Germany	21	1994 - 2005	8[B]	13[C]
Sweden	14	1998 - 2006	14[A]	0
United Kingdom	9	in the 1980s	0	9[B]
Italy	5	1998 and 1999	2[B]	3[C]
Belgium	4	1976, 2004 and 2005	3[B]	1[C]
Austria	2	2007	0	2[A]
Norway	1	1998	1[A]	0
Totals	104		72	32

[A]Isolates from with certainty unvaccinated birds.
[B]Isolates from birds with high suspicion for not being vaccinated.
[C]Isolates from birds with high suspicion for being vaccinated.

Table 2. Results of RFLP analysis of the *TK* gene of *ILTV* field isolates and vaccine strains after *Hae* III-digestion.

ILTV	Number of isolates and vaccines	Clone 1	Clone 2	Clone 3
Isolates				
Switzerland	48	43	5[A]	0
Germany	21	21	0	0
Sweden	14	13	0	1[B]
United Kingdom	9	9	0	0
Italy	5	5	0	0
Belgium	4	4	0	0
Austria	2	2	0	0
Norway	1	1	0	0
Vaccines				
CEO	1	1	0	0
TCO	1	1	0	0
Totals	106	100	5	1

[A]Clone 2 was isolated from non-commercial poultry between 1973 and 1995. The birds were highly suspicious for not being vaccinated.
[B]Clone 3 was isolated from unvaccinated non-commercial poultry in 2004.

Table 3. Summarized results of the positions of nucleotide and amino acid changes and the restriction sites for *Hae* III of the sequences of the isolates CH04, CH95 and S04 and the vaccines CEO and TCO for the *TK* protein.

Clone	Clone 1			Clone 2	Clone 3
Strain sequenced	CH04	CEO	TCO	CH95	S04
GenBank accession number	EU360946	EU360949	EU360950	EU360947	EU360948
Positions of nucleotide changes					
123	T	T	T	C	T
141	G	G	G	A	G
411	A	A	A	A	G
540	T	C	C	C	T
594	A	A	A	C	A
755	C	C	C	T	C
Position of amino acid change					
252	Thr	Thr	Thr	Met	Thr
Positions of restriction sites for *Hae* III (GG:CC)	004:005 108:109 959:960	004:005 108:109 959:960	004:005 108:109 959:960	004:005 108:109 592:593 959:960	004:005 108:109 411:412 959:960

Curriculum vitae

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